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(54) Title: METHOD OF PRODUCING A CHIMERIC PROTEIN

(57) Abstract

A method of producing a chimeric protein from i.e. a plant virus coding for such a protein. The method allows the production of large (i.e. 25 kDa) proteins which assemble with the virus in infected host cells and are arranged on the outer surface of chimeric viruses. A vector for the production of biologically useful proteins in such a manner is also disclosed.

1   METHOD OF PRODUCING A CHIMERIC PROTEIN

2

3   This invention relates to a method of producing a

4   chimeric protein, eg a biologically active protein such

5   as an antibiotic peptide.

6

7   Typical antibiotic peptides include the marginins, 23

8   amino acid-long alpha-helical peptides, originally

9   identified from frog skin, which have significant

10 antibacterial activity; the defensins which combat

11 bacteria, fungi and some enveloped viruses such as

12 herpes simplex virus and HIV; and the protegrins which

13 are 16-18 amino acid-long antibiotic peptides with

14 strong biocidal activity.

15

16 The protegrins form part of an array of antibiotic

17 peptides that are used by mammalian phagocytes to

18 destroy invading pathogens through non-oxidative

19 processes. Typically the protegrins include 4 cysteine

20 residues and form a double-stranded β-sheet structure

21 and show sequence similarity with the antibiotic

22 defensin peptides that are also involved in phagocyte

23 defence responses. The defensins are cationic,

24 cysteine-rich peptides of 29 to 34 amino acids that are

25 formed almost entirely of β-sheet structures and that

2

1   have been shown to have biocidal activity against

2   bacteria, fungi and some enveloped viruses, including

3   herpes simple virus and HIV. Both the protegrins and

4   defensins are expressed in phagocytes as pre-pro-

5   proteins which are cleaved to release the biocidal

6   peptides from the carboxy-terminus of the protein.

7

8   Because of their antibacterial activity it may not be

9   convenient to synthesize these antibiotic peptides by

10   genetic engineering in conventional prokaryotic

11   expression systems. Solution synthesis of large

12   amounts of these peptides with a variety of amino acid

13   modifications may be possible, but is not currently

14   considered commercially viable, since a significant

15   drop in yield occurs in the manufacture of peptides of

16   over 25-30 amino acid residues.

17

18   Eukaryotic expression systems (yeast, insect, animal or

19   plant cells which produce foreign proteins or peptides)

20   may be necessary if there is a need for post-

21   translational modification of the desired protein, but

22   fermentation processes for such eukaryotic expression

23   systems are expensive to maintain, provide little

24   flexibility in terms of scaling the process up to

25   industrial production levels and are very susceptible

26   to contamination. Processing and purification of the

27   desired protein can also be complex and costly.

28

29   The use of plants and benign plant viruses offers an

30   opportunity to produce foreign proteins with minimal

31   host cell contamination, thereby reducing contamination

32   problems which could affect successful achievement of

33   the required regulatory body approval for human or

34   veterinary applications.

35

36   It has been proposed in WO92/18618 to use plant viruses

1   as vector systems for the expression of foreign
2   nucleotide sequnces. WO92/18618 describes the use of
3   a Comovirus (Cowpea Mosaic Virus or CPMV) as an
4   effective vector for such expression and also mentions
5   other spheroidal viruses such as HIV and Picorna-
6   viruses. *Picornaviridae* generally comprise particles
7   of 22-30nm having cubic symmetry; *Comoviridae* have a
8   pair of 28nm particles with a similar symmetry, and HIV
9   is a member of the *Retroviridae* which are generally
10 enveloped 100nm particles containing an icosahedral
11 nucleocapsid.
12
13 One disadvantage of the system disclosed in WO92/18618
14 is that the geometry of the spheroidal viruses
15 precludes large proteins from being produced, since the
16 size and number of chimeric proteins per virus particle
17 (generally 60 for icosahedral virus particles) is
18 limited by the spheroidal geometry of the virus.
19
20 Construction of chimeric proteins in such viruses is
21 also limited to the insertion of the foreign component
22 into a loop in a native virus protein, eg the β-B to β-
23 C loop in VP23 of CPMV, where such insertion does not
24 affect the geometry of the coat protein and/or its
25 ability to self-assemble into a virus particle
26 (virion). As can be appreciated, the size of the
27 peptide which can be tolerated in such an insertion is
28 fairly limited; polypeptides of a maximum of 26 amino
29 acids in length are cited by WO92/18618. Larger
30 polypeptides present in internal insertion sites in
31 coat or capsid proteins of the viruses exemplified may
32 result in disruption of the geometry of the protein
33 and/or its ability to successfully interact with other
34 coat proteins leading to failure of the chimeric virus
35 to assemble. Modified viruses which cannot self-
36 assemble might not infect other host cells and produce

4

1   whole plant infection. This possible lack of ability
2   to spread the infection of the modified virus
3   constitutes a significant disadvantage in the prior
4   system.
5
6   The present invention contemplates the use of benign
7   high copy number rod-shaped viruses, preferably plant
8   viruses such as potato virus X (PVX), to produce
9   foreign protein connected to viral coat protein
10   subunits. When assembled, the virus particles comprise
11   long helical arrays of more than 1000 identical
12   chimeric proteins (which are typically coat protein -
13   foreign protein fusion molecules) per virion.
14   Generally the foreign protein portion will be displayed
15   on the outer surface of the virus particles.
16
17   A suitable proteolytic degradation site (eg elastase or
18   CNBr) may be engineered into the chimeric protein to
19   permit release of the foreign protein portion from
20   purified virus material. Given the size of the foreign
21   protein and the relevant composition of the possible
22   viruses, it is estimated that between 10 and 30% of the
23   total weight yield of virus particle could comprise the
24   foreign protein. Release of the foreign protein by
25   proteolytic cleavage can be a simple purification
26   regime, followed by removal of the residual innocuous
27   plant virus itself. Yields of plant virus up to 5g per
28   kg wet weight of leaf from potato or tobacco are
29   possible and hence the yields of foreign protein could
30   be very substantial.
31
32   If the foreign protein is left attached to the chimeric
33   protein in the virus particle, the whole virus particle
34   can also be used as a vector for expression and
35   presentation of peptide epitopes for vaccination of
36   animals and/or the delivery of therapeutic single-

1 stranded RNA molecules. This may be of utility in the
2 delivery of anti-sense or tripl x nucleotides.
3
4 The present invention provides a method of producing a
5 chimeric protein comprising:
6
7   a. providing a rod-shaped recombinant virus or
8   pseudovirus containing a polynucleotide encoding a
9   chimeric protein having a first (viral) portion
10   and a second (non-viral) portion, the chimeric
11   protein being capable of assembly into a virus
12   particle such that the second portion is disposed
13   on the exterior surface of the assembled virus
14   particle;
15
16   b. infecting a host cell with the virus or
17   pseudovirus; and
18
19   c. allowing replication of the virus or pseudovirus
20   and expression of the chimeric protein in the host
21   cell.
22
23 The term "rod-shaped" as applied herein to viruses
24 includes filamentous or flexuous viruses, which are
25 preferred. It is advantageous to use a virus which is
26 flexuous (ie which can bend easily) since chimeric
27 proteins with large second portions may be able to
28 assemble more easily into virus particles (virions)
29 which are flexuous than those which are rigid. PVX is
30 preferred since it forms a flexuous virion.
31
32 The virus or pseudovirus can preferably assemble in the
33 host cell to produce infective virus particles which
34 comprise nucleic acid and chimeric protein. This
35 enables the infection of adjacent cells by the
36 infective virus or pseudovirus particle and expression

6

1   of the chimeric protein therein.

2

3   The host cell can be infected initially with virus or

4   pseudovirus in particle form (ie in assembled rods

5   comprising nucleic acid and protein) or alternatively

6   in nucleic acid form (ie RNA such as viral RNA; cDNA or

7   run-off transcripts prepared from cDNA) provided that

8   the virus nucleic acid used for initial infection can

9   replicate and cause production of whole virus particles

10 having the chimeric protein.

11

12 The term "pseudovirus" as used herein means a virus-

13 derived nucleic acid sequence optionally assembled into

14 particles and having an incomplete viral genome as

15 compared to wild-type virus but retaining sufficient

16 viral genes to allow replication and assembly of the

17 pseudovirus. The virus or pseudovirus may contain

18 genetic material foreign to the wild-type virus.

19

20 Optionally, the virus or pseudovirus can be purified

21 from the host cell in order to concentrate the chimeric

22 protein, ie by polyethylene glycol precipitation and/or

23 density gradient centrifugation.

24

25 Optionally, the method may include the step of

26 separating a protein derived from the second portion

27 from the remainder of the chimeric protein after the

28 virus or pseudovirus has been purified from the host

29 cell.

30

31 A linker peptide can be incorporated between the first

32 and second portions and may have the function of

33 spacing the two portions from one another, reducing

34 stearic restrictions. Optionally the linker peptide

35 may contain a proteolytic or chemical cleavage site.

36

1   The term "proteolytic or chemical cleavage site" refers
2   to a short sequence of amino acids which is
3   recognisable and subsequently cleavable by a
4   proteolytic enzyme or chemical means. Suitable
5   proteolytic enzymes include trypsin, pepsin, elastase
6   and the like. Alternatively the proteolytic or
7   chemical cleavage site may be a site which is
8   vulnerable to cleavage by other means, for example by
9   addition of chemicals such as cyanogen bromide (CNBr)
10   or acids or by shear. Preferably, the proteolytic or
11   chemical cleavage site is an elastase cleavage site,
12   but other suitable proteolytic cleavage sites can be
13   used with corresponding enzymes.
14
15   The protein derived from the second portion may be
16   separated from the remainder of the chimeric protein
17   before assembly of the virus particle, eg during
18   expression of the genetic material coding for the
19   chimeric protein, or during assembly of the chimeric
20   protein into a virus particle. In this embodiment the
21   host cell will contain free protein derived from the
22   second portion. This embodiment can be useful when
23   expression of very large proteins derived from the
24   second portion is desired. In such an embodiment, the
25   proteolytic or chemical cleavage site may be selected
26   to cleave automatically in a virally-infected host
27   cell.
28
29   The term "proteolytic or chemical cleavage site" may
30   thus also include sequences that cleave automatically
31   such as the FMDV (Foot and Mouth Disease Virus) 2A
32   protease.
33
34   The proteolytic or chemical cleavage site may be an
35   integral part of either the first or second portion.
36   Hence either/or both of the portions may include an

8

1   integral proteolytic or chemical cleavage site.

2

3   Thus the present invention also provides a method f

4   producing a chimeric protein as defined above, wherein

5   the protein derived from the second portion is purified

6   directly from the host cell after expression.

7

8   The second portion and/or the protein derived therefrom

9   may be relatively large eg over 10kDa. Proteins of 25-

10   30 kDa are suitable for production by the method and

11   even proteins up to 60-70 kDa have been shown to be

12   produced by the method of the invention.

13

14   The first (viral) portion of the chimeric protein may

15   be any protein, polypeptide or parts thereof, derived

16   from a viral source including any genetically modified

17   versions thereof (such as deletions, insertions, amino

18   acid replacements and the like). In certain

19   embodiments the first portion will be derived from a

20   viral coat protein (or a genetically modified version

21   thereof). Mention may be made of the coat protein of

22   Potato Virus X as being suitable for this purpose.

23   Preferably the first portion has the ability to

24   aggregate into particles by first-portion/first portion

25   association. Thus, a chimeric protein molecule can

26   assemble with other chimeric protein molecules or with

27   wild-type coat protein into a chimeric virion.

28

29   In a preferred embodiment of the invention the particle

30   is derived from a potyvirus or even more preferably a

31   potexvirus such as PVX, and in such an embodiment, the

32   second portion is preferably disposed at or adjacent

33   the N-terminus of the coat protein. In PVX, the N-

34   terminus of the coat protein is believed to form a

35   domain on the outside of the virion.

36

1   The second portion of the chimeric protein may be any
2   protein, polypeptide or parts thereof, including any
3   genetically modified versions thereof (such as
4   deletions, insertions, amino acid replacements and the
5   like) derived from a source other than the virus from
6   which the first portion is derived. In certain
7   embodiments the second portion or the protein derived
8   therefrom is a biologically active or useful molecule.
9   The second portion or the protein derived therefrom may
10   also be a diagnostic reagent, an antibiotic or a
11   therapeutic or pharmaceutically active agent.
12   Alternatively the second portion or the protein derived
13   therefrom may be a food supplement.
14
15   In an alternative embodiment, the second portion or the
16   protein derived therefrom may be an indicator protein
17   chosen for its ability to indicate the location of the
18   chimeric protein or of the virus particle. Such an
19   example is the 25kDa jellyfish green fluorescent
20   protein.
21
22   The polynucleotide coding for the second (non-viral)
23   portion may be inserted into an appropriate restriction
24   site in the viral genome. The restriction site adopted
25   for such insertion may be naturally occurring in the
26   viral genome or artificially constructed therein and
27   the polynucleotide coding for the second portion may be
28   ligated therein by conventional means. General
29   techniques for cloning of foreign nucleic acid and
30   construction of chosen restriction sites is
31   comprehensively described in the art and is within the
32   scope of the skilled person.
33
34   It is preferred that the polynucelotide coding for the
35   second portion is inserted at or adjacent a terminus of
36   the polynucleotide coding for the first portion, such

10

1   that upon translation the chimeric protein has the
2   first portion at one nd and the second portion at the
3   opposite end. It is not necessary for the first
4   portion to comprise a whole virus coat protein, but
5   this remains an option.
6
7   The virus particle may be formed by the assembly of
8   chimeric proteins only or by the mixed assembly of
9   chimeric proteins together with some unmodified or less
10 modified forms of the naturally occurring wild-type
11 coat protein which forms the basis of the first
12 portion. For a mixed virus particle of the latter
13 type, there must be present polynucleotide(s) encoding
14 the chimeric protein and the naturally occurring coat
15 protein. The appropriate protein-coding sequences may
16 be arranged in tandem on the same molecule. An
17 alternative would be co-infection (for example of
18 mutually dependant defective viruses or pseudoviruses)
19 of two or more viruses or pseudoviruses, or infection
20 by chimeric virus of a host cell or whole organism
21 (such as a plant) which expresses such a protein
22 intrinsically.
23
24 An advantage is gained by using a virus which forms a
25 particle with a relatively high pitch of helix. PVX
26 has a pitch of 3.4nm and is to be preferred over
27 viruses with a lower pitch. Virus particles with
28 higher pitches may be able to accommodate larger
29 protein insertions on their surfaces since their coat
30 proteins assemble with more space between them than
31 coat proteins of viruses with lower pitches.
32
33 A virus or pseudovirus genetically modified to express
34 the chimeric protein forms a further aspect of the
35 present invention, as does any host cell infected with
36 such a virus or pseudovirus.

1   Preferably, the host cell used to replicate the virus
2   or pseudovirus is a plant cell where the virus is a
3   plant virus, although insect cells, mammalian cells and
4   bacteria can be used with viruses which will replicate
5   in such cells.
6
7   While modifications and improvements may be
8   incorporated without departing from the scope of the
9   invention, embodiments will now be described by way of
10   the following examples and with reference to the
11   accompanying drawings in which:
12
13   Fig 1a shows the structure of a gene for a
14   chimeric protein and of the overcoat vector
15   pTXS.L2a-CP for use in the present invention;
16   Fig 1b is a schematic diagram showing the major
17   features of plasmids useful in the methods of the
18   present invention;
19   Fig 2 shows a western blot of wild type and
20   chimeric protein taken from leaves of a plant
21   infected by a wild-type and a chimeric virus;
22   Fig 3 a, b, c and d show leaves of plants infected
23   with recombinant virus;
24   Fig 4 a, b, c, d and e are micrographs
25   illustrating the subcellular distribution of
26   chimeric protein expressed from chimeric virus
27   nucleic acid;
28   Fig 5 is an electron micrograph showing
29   aggregation and immuno-gold labelling of
30   chimeric viruses;
31   Fig 6 a, b and c are electron micrographs of
32   negatively-stained chimeric viruses; and
33   Fig 7 is a photograph of a N benthamiana leaf
34   systemically infected with a chimeric virus.
35
36   EXAMPLE 1

12

1   A general strategy for the production of large
2   quantities of recombinant proteins is given below using
3   PVX as an example. A similar strategy could be
4   employed for other flexuous filamentous or rod-shaped
5   viruses. A cDNA clone of potato virus X is first
6   modified to produce fusion proteins between the viral
7   coat protein and proteins with biological activity or
8   other commercial applications. The feasibility of this
9   approach has been demonstrated as described below by
10   creating a translational fusion between the green
11   fluorescent protein (25 kDa) of *Aequorea victoria* (1)
12   and the PVX coat protein (also around 25 kDa).
13   Functional chimeric viruses have also been made which
14   are able to express recombinant genes encoding fusions
15   between the PVX coat protein and the kanamycin
16   resistance protein Neomycin phosphotransferase (25 kDa)
17   and between PVX coat protein and the more complex
18   enzymes β-galactosidase (10-13 kDa) and β-glucuronidase
19   (68 kDa) respectively.
20
21   The green fluorescent protein (GFP) from *A. victoria*
22   (1) is a reporter of gene expression in heterologous
23   systems (3-6). GFP has an advantage over other marker
24   proteins in that it can be detected non-invasively,
25   without any requirement for exogenous substrates or co-
26   factors (3) since it fluoresces intrinsically without a
27   requirement for exogenous substrate. In addition,
28   fluorescence of GFP is retained in fusion proteins
29   allowing the subcellular localization of fusion
30   proteins (4).
31
32   PCR-mutagenesis of a full-length cDNA copy of the
33   potato virus X genome can be performed to create a
34   synthetic coding sequence comprising the gene coding
35   for the protein of interest, the foot and mouth disease
36   virus 2A protease gene, and the potato virus X coat

13

1   protein gene.   The PVX genome is contained within the
2   known plasmid pTXS (Fig. 1, reference 25).
3
4   When reassembled the modified cDNA copy of the viral
5   genome can be used as a template to synthesize *in vitro*
6   run-off transcripts.   Inoculation of transcripts to
7   plants can be performed by manual abrasion of
8   carborundum coated leaves of either *Nicotiana*
9   *clevelandii* or *N benthamiana*.
10
11 When the above approach was followed using PVX modified
12 to express GFP-CP fusion protein, between two and three
13 days post inoculation the presence of fluorescent
14 regions in the virus infected plants could be observed
15 by eye on inoculated leaves by viewing plants under
16 ultraviolet light.   At about ten days post inoculation
17 GFP-mediated fluorescence was detected in systemic
18 (non-inoculated) leaf tissue (Figure 7).   This
19 fluorescence was specific to the green fluorescent
20 protein and was not observed on control plants
21 inoculated with wild-type PVX.
22
23 Electron microscopic analysis of viral particles showed
24 a clear increase in particle width in plants infected
25 with the GFP-CP containing virus compared with
26 particles isolated from plants infected with wild-type
27 PVX (Figure 6).
28
29 In the strategy used above, foreign proteins were
30 expressed by fusing them to the amino-terminus of the
31 PVX coat protein.   However other sites may be possible,
32 eg carboxy-terminus surface loops on some other rod-
33 shaped or filamentous viruses.
34
35 Data from previous studies suggest that fusion of the
36 proteins to the amino t rminus of the PVX coat protein

14

1   is most likely to be successful. Biochemical,

2   immunological and tritium bombardment data suggest a

3   model for the structure of the PVX coat protein (10) in

4   which the N-terminal 33 amino acids form a domain of β-

5   sheet on the outside of the virion.   In contrast, the

6   C-terminus of the PVX coat protein, which also forms

7   part of a β-sheet structure, is inaccessible from the

8   outside of the virion and deletions within it do not

9   permit the virus to infect plants systemically.

10

11   As an additional optional strategy, the foot and mouth

12   disease virus (FMDV) 2A protease sequence (12) can be

13   positioned between the foreign and coat protein

14   sequences. The FMDV 2A protease is a short (19 amino

15   acid) peptide which acts in cis to cleave the FMDV

16   polyprotein in a co-translational mechanism. This

17   protease has been shown to effect the cleavage of

18   synthetic polyproteins both in vitro and in vivo (13).

19   The inclusion of the 2A protease sequence between the

20   GFP and coat protein can generate a mixed pool of

21   fusion and cleaved proteins in virus infected cells.

22   The presence of free coat protein, generated by 2A

23   protease mediated cleavage, may circumvent this problem

24   by allowing assembly of virions composed of both free

25   (ie cleaved) and fused coat protein subunits.

26

27   The formation of virions is an absolute requirement of

28   PVX for systemic infection of plants (15). The

29   demonstration herein that GFP-coat protein fusions do

30   assemble into virions (Fig 7) and spread indicates that

31   the size of GFP (25kDa) does not interfere with virion

32   assembly. Fusion proteins which fail to assemble due

33   to size or other constraints can be produced in

34   constructs carrying the FMDV 2A protease, or in plants

35   which are modified to express wild-type coat protein

36   for the particular virus used. The sequence of the 2A

15

1   protease peptide can be modified to increase or
2   decrease the efficiency of co-translational cleavage.
3
4   EXAMPLE 2
5   This example describes a modified form of PVX which
6   expresses a chimeric gene encoding a fusion between the
7   *Aequorea victoria* green fluorescent protein and the PVX
8   coat protein and assembles into virions that are over
9   twice the diameter of wild-type PVX. The modified
10 virus moves from cell-to-cell and systemically. The
11 example demonstrates the potential of fusions between
12 non-viral protein and virus coat protein for production
13 of high levels of non-viral proteins in plants.
14
15 The plasmids used in this work were derived essentially
16 from the plasmid pTXS which contains the PVX genome and
17 a T7 promoter (described in 25). Fig 1b shows the
18 following main features of the plasmids: the virus RNA-
19 dependent RNA polymerase gene (RdRp); virus genes
20 encoding movement proteins (M1, M2, M3); the virus coat
21 protein gene (CP); promoters from T7 bacteriophage (T7)
22 or for the 35S RNA of CaMV (CaMV35S); the
23 transcriptional terminator of the nopaline synthase
24 gene of *Agrobacterium tumofaciens* and various
25 restriction enzyme sites.
26
27 The plasmid pCXA3 was constructed by transfer of the
28 PVX cDNA from pTXS into the plasmid pBl220.5 between
29 the CaMV 35SRNA promoter and the nopaline synthase gene
30 terminator. The plasmid pBl220.5 is similar to the
31 plasmid pBl221.1 but without the GUS gene (described in
32 27). The junction between the promoter and the PVX
33 cDNA was modified by oligonucleotide directed
34 mutagenesis to the sequence
35 (5')gatttggagagga*gaaaactaaacca(3') in which * denotes
36 the most 3' non-transcribed position in the promoter

16

1   sequence and th most 5' transcribed position in the
2   viral genome (28). Construction of the pVX201 vector
3   from pCXA3 and pPC2S exploited unique restriction sites
4   at positions 4945 (ApaI) and 6302 (XhoI) of the PVX
5   cDNA (25).

6

7   GFP cDNA was PCR-amplified with primers
8   (5')gcca<u>atcgat</u>catgagtaaaggag(3') on the positive strand
9   and (5')ggaa<u>gtcgac</u>acatttatttg(3') from the negative
10 strand. The bold type represents the initiation and
11 termination codons of the GFP gene (29). The
12 underlined type represents ClaI and SalI sites used to
13 introduce the PCT-amplified sequence into pPVX201 to
14 generate pPVX204. The plasmid pTXS.GFP was made by
15 substitution of the region of pPVX204 containing the
16 GFP sequence into the homologous region of pPC2S.

17

18 The plasmid pTXS.GFP carries a full-length cDNA copy of
19 the potato virus X (PVX) genome into which the GFP gene
20 has been inserted. Inoculation of plants with
21 transcripts synthesized *in vitro* from pTXS.GFP results
22 in the expression of free GFP in infected cells (5).
23 We prepared a derivative of pTXS.GFP, pTXS.GFP-CP, to
24 create a translational fusion between the
25 carboxyterminus of the GFP and the amino-terminus of
26 the PVX coat protein (CP). pTXS.GFP was used as a
27 template to produce the GFP-2A-CP fusion gene by
28 overlap extension PCR using flanking oligonucleotides
29 complementary to the PVX genome and mutagenic
30 oligonucleotides to incorporate the 2A protease coding
31 sequence. Amplified product was subcloned into
32 pTXS.GFP as a 1.5 kbp fragment using the unique
33 restriction sites ClaI and XhoI to give pTXS.GFP-CP.
34 Fig. 1a shows a schematic representation of viral cDNAs
35 used to synthesize infectious run-off transcripts for
36 the GFP-2A-CP fusion gene. The predicted Mrs of the

1   four viral proteins common to all constructs ar
2   indicated (K=kD). The polypeptide chain lengths of the
3   CP, GFP and 2A protease (2A) enclosed by the constructs
4   are shown. The bars indicate the position of the
5   subgenomic promoter for the CP. TXS=wild-type PVX;
6   TXS.GFP=PVX modified to express free GFP from a
7   duplicated subgenomic promoter; TXS.GFP-CP=PVX modified
8   to express the GFP-2A-CP fusion protein.
9
10   Because the GFP and PVX CP are of similar sizes, having
11   molecular weights of 26.9 kD and 25.1 kD respectively,
12   it was expected that in a homogenous population of
13   fusion protein steric effects would prevent virion
14   formation. Assembly of fusion protein into virions
15   might be facilitated by the presence of a pool of free
16   CP. Therefore the GFP and CP nucleotide sequences in
17   pTXS.GFP-CP were separated by sequence coding for
18   sixteen amino acids from the foot-and-mouth disease
19   virus (FMDV) 2A peptide. The 2A region of FMDV
20   mediates a primary (co-translational) processing event
21   between the 2A and 2B regions of the FMDV polyprotein
22   (12) that results in inhibition of peptide bond
23   formation (13).
24
25   In vitro run-off transcripts (14), synthesized from
26   pTXS.GFP and pTXS.GFP-CP (plasmids were linearized with
27   Spe 1 prior to in vitro transcription reactions as
28   described in reference 14), were infectious when
29   inoculated to plants; virus derived from transcript-
30   infected plants is subsequently referred to as PVX.GFP
31   and PVX.GFP-CP respectively.
32
33   Following inoculation of either Nicotiana clevelandii
34   or N. benthamiana, both PVX.GFP and PVX.GFP-CP caused
35   the development of green fluorescent regions which were
36   first detectable by eye under UV illumination b tween

18

1   two and three days post inoculation (Fig. 3A, C).

2   Subsequent long-distance movement of the virus to

3   developing leaves led to the appearance of green

4   fluorescence in systemically infected leaves (Fig. 3B,

5   D). The rate at which fluorescent regions spread on

6   inoculated leaves was slower in PVX.GFP-CP infected

7   plants than PVX.GFP infected plants and the appearance

8   of fluorescence in systemically infected leaves was

9   delayed in plants infected with PVX.GFP-CP compared

10 with PVX.GFP infected plants.

11

12 Fig. 3 shows leaves of *N. benthamiana* infected with

13 either PVX.GFP (A, B) or PVX.GFP-CP (C,D). Leaves were

14 viewed under UV illumination (365 nm) generated from a

15 Blak Ray B100-AP lamp (Ultra-Violet Products) and

16 photographed using a Wratten 58 filter to eliminate

17 chlorophyll auto-fluorescence. The pattern of virus

18 spread in both cases is similar. A and C identify

19 inoculated leaves showing the development of

20 characteristic circular legions. B and D identify

21 systemically infected leaves showing fluorescence

22 associated predominantly with the leaf veins. The

23 developing leaf (D) was undergoing the sink-source

24 transition (20) resulting in lack of virus movement

25 into the apical portion of the leaf.

26

27 Fig 4a is a confocal fluorescence image of a

28 systemically infected leaf in transverse section

29 showing the location of PVX.GFP-CP containing

30 viroplasms within individual cells of the leaf. 4b is

31 a bright field image of section shown in (A) showing

32 the typical arrangement of epidermis (E), palisade (P)

33 and mesophyll(M) cells. A vascular bundle (B) is also

34 present (scale=50 μm). 4c is a confocal image of

35 palisade cells from a leaf systemically infected with

36 PVX.GFP-CP showing the GFP-containing viroplasms (V)

19

1   assembled into cage-like structures (scale=5 μm).   4d
2   shows a leaf trichome systemically infected with
3   PVX.GFP, in which the GFP is associated with the
4   nucleus (N) and the cytoplasm.   4e shows a leaf
5   trichome systemically infected with PVX.GFP-CP, in
6   which the GFP is predominantly targeted to viroplasms
7   (V) within individual trichome cells (scale=10 μm).
8
9   In systemically infected (ie non-inoculated) leaves
10   both PVX.GFP and PVX.GFP-CP moved from the phloem into
11   surrounding bundle sheath and mesophyll cells and
12   eventually into the epidermis (Fig. 4A, B).   Under the
13   confocal microscope transverse sections of the
14   systemically infected leaves showed that in PVX.GFP-CP
15   infected cells green fluorescence was detected
16   predominantly in viroplasms, cytoplasmic structures
17   comprising aggregated viral particles that often
18   appeared as continuous cage-like structures within the
19   cell (Fig 4C, 5).   By contrast, in PVX.GFP infected
20   cells, the green fluorescence was associated with
21   nuclei and showed a relatively uniform distribution
22   throughout the cytoplasm.   This difference in the
23   subcellular distribution of the GFP was seen clearly in
24   leaf trichome cells (Fig. 4D, E).
25
26   The distribution of fluorescence suggested that the
27   majority of GFP produced in PVC.GFP-CP infected plants
28   was still fused to the CP and that these fusion
29   proteins were assembling into virions, which
30   subsequently formed viroplasms.
31
32   Western blotting of protein extracts from inoculated *N.*
33   *clevelandii* leaves, probed with CP specific antiserum
34   (16), showed that most of the immunoreactive protein in
35   PVX.GFP-CP infected plants comprised the fusion
36   protein.   Protein extracts were prepared by grinding

20

1   leaf tissue in two volumes (w/v) protein extraction
2   buffer (15). An equal volume of 2x SDS load buffer was
3   added and the extracts were boiled for two minut s.
4   Proteins were electrophoresed, blotted to
5   nitrocellulose and probed with rabbit polyclonal anti-
6   PVX CP antiserum as described previously (16) .Fig 2
7   illustrates the data obtained. Protein was prepared
8   from mock inoculated control plants (lane 2), or from
9   plants inoculated with in vitro transcripts synthesized
10   from plasmid DNAs (TXS=lane 1; TXS.GFP-CP=lane 3;
11   TXS.GFP=lane 4). Mrs of native CP, the GFP-2A-CP
12   fusion protein and CP released by 2A protease mediated
13   cleavage are 25.1, 53.2 and 24.8 kD respectively. The
14   Mrs of standards are shown to the left of Fig 2 in kD.
15
16   The low level of smaller immunoreactive protein
17   detected in PVX.GFP-CP infected tissue is assumed to
18   result from processing of the fusion protein mediated
19   by the FMDV 2A peptide rather than from contamination
20   with virus deletion mutants as similar ratios of fusion
21   to free protein were observed in all other samples
22   analyzed and RT-PCR analysis of the same samples used
23   for protein analysis showed no evidence of deleted
24   forms of the viral genome (17). In addition when blots
25   were probed with GFP specific antiserum the ratio of
26   free protein to fusion protein was the same as that
27   observed using anti-CP antiserum (17).
28
29   In order to determine the subcellular location of the
30   viral CP ultrathin sections of inoculated leaves were
31   prepared for immuno-gold labelling, using a polyclonal
32   antibody to the PVX CP. Leaf tissues were fixed and
33   embedded in Araldite (TM) resin for immuno-gold
34   labelling as described previously (17). Ultrathin
35   sections on nickel grids were labelled using polyclonal
36   rabbit antiserum to the PVX CP followed by goat anti-

1   rabbit gold conjugate (GAR-15 nm, Amersham

2   International).   Aggregation of the filamentous

3   virions into viroplasms is marked with arrows in Fig 5.

4   Dense gold labelling was predominantly associated with

5   the viroplasms in both PVX.GFP and PVX.GFP-CP infected

6   cells.   The pattern of virus aggregation seen in the

7   electron microscope for both PVX.GFP-CP (Fig. 5) and

8   PVX.GFP was remarkably similar to the cages of

9   viroplasm seen with PVX.GFP-CP under the confocal

10 microscope (Fig. 4c).

11

12 For negative staining, virus particles were trapped

13 from virus infected sap extracts by immuno-sorbent

14 electron microscopy (18) using anti-PVX CP antiserum,

15 and stained with 2% sodium phosphotungstate (pH 7).

16 Analysis of negatively stained virus samples under the

17 electron microscope revealed that PVX.GFP-CP virions

18 were decorated along their length with globular

19 extensions (Fig. 6a,b).   Fig 6c shows negatively

20 stained virus rods isolated from PVX.GFP infected

21 plants (scale=50 nm).   Differences in virion diameter

22 are seen most clearly where virions are aligned in

23 parallel (a and c, large darts).   In Fig 6b small

24 globular extensions (small darts) are apparent along

25 the length of the PVX.GFP-CP virus (scale=25 nm).   The

26 PVX.GFP-CP virions had a mean diameter of 29.7 nm, more

27 than twice the diameter of PVX.GFP virions (12.6 nm;

28 Fig. 6c).

29

30 A modified form of PVX.GFP-CP, in which the FMDV 2A

31 peptide sequence carries three amino acid

32 substitutions, introduced to prevent processing of the

33 polyprotein, was unable to move from cell-to-cell and

34 did not give rise to fluorescent viroplasms.

35 Infections with this mutant were restricted to single

36 epidermal cells and fluorescence was detected uniformly

1   throughout the cytoplasm and in association with
2   nuclei, as observed for PVX.GFP infections (17),
3   suggesting that the presence of free CP is essential
4   for either initiation of elongation of virions.
5
6   The fluorescence generated by the GFP attached to
7   virions was intense, allowing rapid detection of viral
8   aggregates within individual living cells.
9   Furthermore, confocal microscopy allowed the
10   noninvasive imaging of the pathway of cell-to-cell
11   movement of virus-GFP constructs, pinpointing the
12   specific cell types in which virus accumulated. For
13   confocal imaging leaves were excised from the plant and
14   sectioned transversely into 200 μm slices using a
15   vibrotome. The sections were immediately mounted in
16   water and viewed under a Bio-Rad MRC 1000 confocal
17   laser scanning microscope at an excitation wavelength
18   of 488 nm using a krypton-argon laser.
19
20   Previous descriptions of assembly competent plant RNA
21   viruses carrying CP extensions have involved small
22   oligopeptide fusions (19). The data presented in this
23   example suggest that the system described could be used
24   for the production of proteins that are at least as
25   large as the viral CP of PVX.
26
27   The strategy described to generate GFP-coat protein
28   fusions can be easily applied to proteins other than
29   GFP. We modified the plasmid pTXS.GFP-CP which carries
30   the GFP-2A-CP fusion protein gene to enable the facile
31   insertion of novel coding sequence as a fusion to the
32   2A-CP cassette. This modified plasmid, pTXS.L2a-CP
33   shown in Fig. 1a (deposited under No NCTC 12918 at the
34   National Collection of Type Cultures at 61 Colindale
35   Avenue, London NW9 5HT on 18 October 1995) carries a
36   series of unique restriction enzyme recognition sites

1   (ClaI, EgaI, SmaI, EheI) or polylinker that replaces

2   the GFP coding sequence of pTXS.GFP-CP. By digesting

3   the vector pTXS.L2a-CP at one or more of the polylinker

4   restriction enzyme sites it is possible to insert the

5   coding sequence for any given protein such that a

6   fusion protein gene is created comprising the novel

7   gene, the FMDV 2A peptide and the PVX coat protein as a

8   translational fusion.

9

10 The plasmid vector pTXS.L2a-CP was prepared by PCT-

11 based mutagenesis of the plasmid pTXS.GFP-CP using

12 standard techniques (26). The oligonucleotide 2aL5'

13 was annealed to the primer 2aL3' and extended with T4

14 DNA polymerase.

15

16 The sequence of primers used was

17

18 2aL5': 5' TCG GCC GTC CCG GGG GCG 3'

19 ||| ||| ||| ||| ||| |||

20 2aL3': 3' AGC CGG CAG GGC CCC CGC GGT TAA AAC TGG AAG

21

22   AAT TCG AAA 5'

23

24 The extended product was gel purified and cloned into

25 the plasmid M13RK8.2 (30). An Eag 1/ Afl 11 fragment

26 was excised from the resulting plasmid and cloned

27 between the same sites of the plasmid pTXS.GFP-CP in

28 place of the GFP gene.

29

30 Thus, the nucleotide sequence of the new linker in

31 pTXS.L2a-CP is

32   ClaI   EagI   SmaI   EheI

33

34   /_____\ /_____\   /_____\ /___\

35 Nts: AT CGA TCC GGC CGT CCC GGG GGC GCC AAT TTT

36 Amino acids: Pro Gly Gly Ala Asn Phe

24

1   Inserti n of foreign genes into th pTXS.P-CP
2   polylinker are m st easily perform d by PCR
3   amplification of the foreign gene using
4   oligonucleotides designed to incorporate appropriate
5   restriction enzyme recognition sites at the 5'- and 3'-
6   termini of the foreign coding sequence such that the
7   gene for the synthetic polyprotein comprises a single
8   open reading frame. We have demonstrated the utility
9   of this approach using the gene encoding neomycin
10   phosphotransferase (NPT) which confers resistance to
11   the antibiotic kanamycin and is present in most
12   commercially available plasmids as a selection tool.
13   The 0.73 kb (NPT) coding sequence was inserted into the
14   polylinker of pTXS.P-CP to give the plasmid pTXS.NPT-
15   CP. Transcripts synthesized in vitro from the
16   pTXS.NPT-CP template were infectious on plants and the
17   virus moved both locally and systemically. Assembly of
18   PVX.NPT-CP virions results in "overcoat" virus
19   particles carrying the NPT protein on the surface of
20   the virions.
21
22   The advantages of the invention are as follows:
23
24   (i) Standard purification procedures exist (eg
25   polyethylene glycol precipitation and centrifugation)
26   for these highly stable virus particles to remove plant
27   proteins and cellular debris and to give an extremely
28   pure suspension of plant virus particles. Plant
29   viruses are innocuous to humans, ingestion experiments
30   have already revealed that they pass straight through
31   the intestine undamaged.
32
33   (ii) By attaching the foreign protein to each (or a
34   subset of) coat protein subunits optionally with a
35   suitable cleavage-sensitive linker sequence will allow,
36   following virus purification from the infected plant

25

1   sap, foreign protein to be released into free solution
2   simply by incubation with the appropriate proteolytic
3   enzyme. The released virus particles remain stable and
4   of high molecular weight so that they can be separated
5   from the short peptide either by simple dialysis
6   procedures (continuous flow type), or by differential
7   centrifugation or selective precipitation.
8
9   (iii) Yields of cleaved foreign protein from such a
10   system could reach 50% or more of the total weight of
11   virus recovered. Each helical virus particle has 95%
12   of its weight as coat protein, and each coat protein
13   subunit has a molecular weight of approximately 25 kD.
14   In the model system already developed the green
15   fluorescent protein also has a molecular mass of
16   approximately 25 kD. Yields of potato virus X can be
17   extremely high (up to 5 gm/kg wet weight of infected
18   leaf after several weeks).
19
20   (iv) The flexibility of scale that can be achieved in
21   plants is also attractive in terms of reducing the cost
22   of protein production and avoids the need for high
23   level capital investment such as in animal or microbial
24   cell culture facilities.
25
26   (v) The use of set-aside land and/or discredited crops
27   such as tobacco for the alternative production of
28   highly prized, pharmaceutically active proteins would
29   lead to considerable added value in the peri-
30   agricultural sector.
31

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incorporated herein by reference:

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23

29

1   Claims:

2

3   1   A method of producing a chimeric protein, the
4   method comprising:

5

6   a   providing a rod-shaped recombinant virus or
7   pseudovirus containing a polynucleotide encoding a
8   chimeric protein having a first (viral) portion
9   and a second (non-viral) portion, the chimeric
10   protein being capable of assembly into a virus
11   particle such that the second portion is disposed
12   on the exterior surface of the assembled virus
13   particle;

14

15   b   infecting a host cell with the virus or
16   pseudovirus; and

17

18   c   allowing replication of the virus or pseudovirus
19   and expression of the chimeric protein in the host
20   cell.

21

22   2   A method according to claim 1, wherein the
23   chimeric protein assembles into a virus particle.

24

25   3   A method according to claim 1 or claim 2, wherein
26   the virus or pseudovirus is subsequently purified from
27   the host cell.

28

29   4   A method according to claim 2 or claim 3,
30   including the step of cleaving the second portion or a
31   protein derived therefrom from the first portion after
32   purification of the virus or pseudovirus from the host
33   cell.

34

35   5   A method according to any preceding claim, wherein
36

1   a linker peptide is incorporated between the first and

2   second portions.

3

4   6   A method according to any preceding claim, wherein

5   a proteolytic cleavage site is incorporated on one of

6   or between the first and second portions.

7

8   7   A method according to claim 1, wherein the first

9   and second portions are separated from one another

10 before or during assembly of the virus particle, such

11 that the host cell contains free protein derived from

12 the second portion.

13

14 8   A method according to any preceding claim, wherein

15 protein derived from the second portion is purified

16 from the host cell after replication.

17

18 9   A method according to any preceding claim, wherein

19 the virus or pseudovirus is derived from a plant virus.

20

21 10   A method according to any preceding claim, wherein

22 the virus or pseudovirus is derived from potato virus

23 X.

24

25 11   A method according to any preceding claim, wherein

26 the second portion is disposed at or adjacent the N-

27 terminus of the viral coat protein.

28

29 12   A method according to any preceding claim, wherein

30 the second portion is a diagnostic reagent, an

31 antibiotic, a therapeutic or pharmaceutically active

32 agent, a vaccine or a food supplement.

33

34 13   A method according to any preceding claim, wherein

35 the virus or pseudovirus particle comprises a mixture

36 of chimeric protein and wild-type coat protein.

1   14   A method according to any preceding claim, wherein
2   the virus or pseudovirus particle has a relatively high
3   pitch of helix.
4
5   15   A method according to claim 12, wherein the pitch
6   of the helix is more than 2nm.
7
8   16   A method according to any preceding claim, wherein
9   the virus or pseudovirus is flexuous.
10
11   17   A method according to any preceding claim, wherein
12   the host cell is infected with virus or pseudovirus in
13   particle form.
14
15   18   A method according to any one of claims 1-16,
16   wherein the host cell is infected with virus or
17   pseudovirus in nucleic acid form.
18
19   19   A method according to any preceding claim, wherein
20   the second portion or a peptide derived therefrom has a
21   molecular weight in excess of 10 kDa.
22
23   20   A virus or pseudovirus genetically modified to
24   express a chimeric protein, the chimeric protein having
25   a first (viral) portion linked to a second (non-viral)
26   portion, the chimeric protein being capable of self-
27   assembly into a virus particle so that the second
28   portion is disposed on the exterior surface of the
29   assembled virus particle.
30
31   21   A host cell, plant, animal or insect infected
32   with a virus or pseudovirus according to claim 20.
33
34   22   A polynucleotide capable of producing a virus or
35   pseudovirus according to claim 20.
36

32

1   23   A chimeric protein produced by a method according
2   to any one of claims 1-19.
3
4   24   The plasmid pTXS.L2a-CP as deposited under No NCTC
5   12918 on 18 October 1995 at the National Collection of
6   Type Cultures.
7
8
9

FIGURE 1a
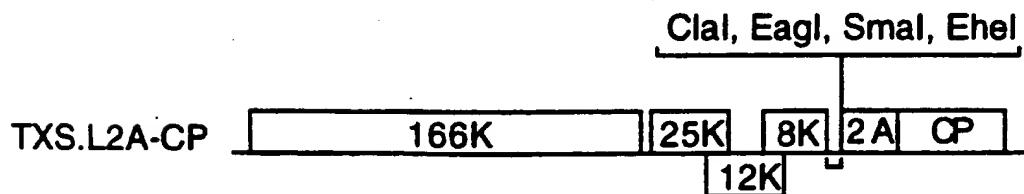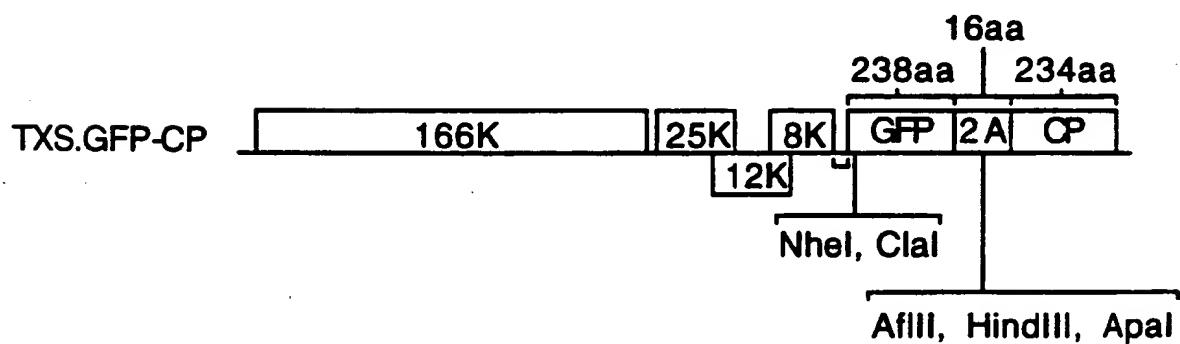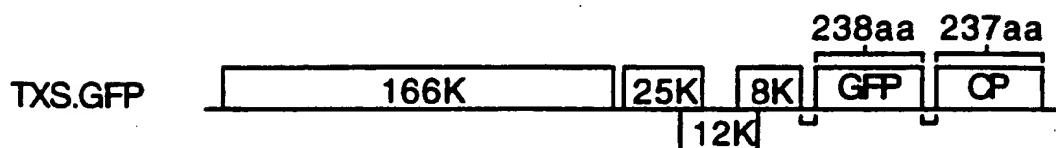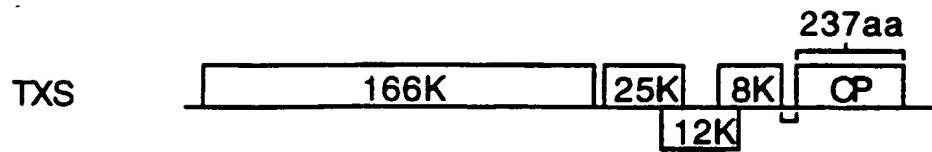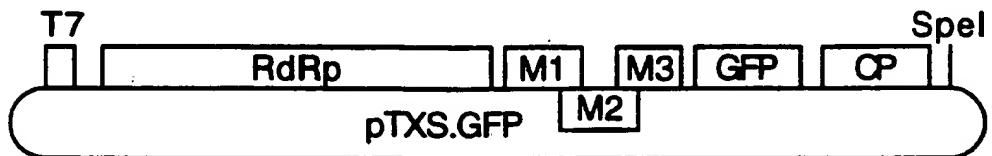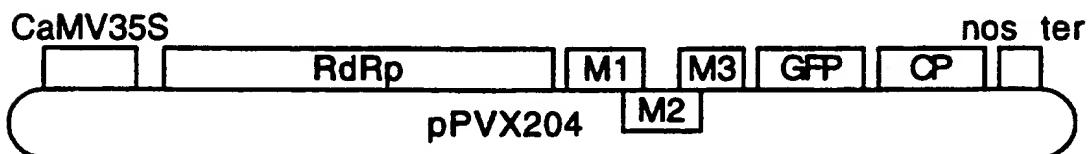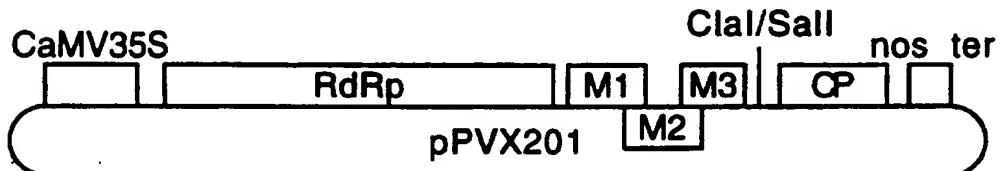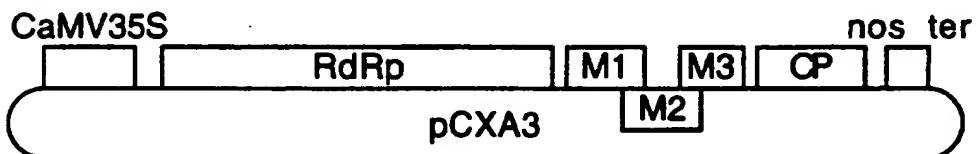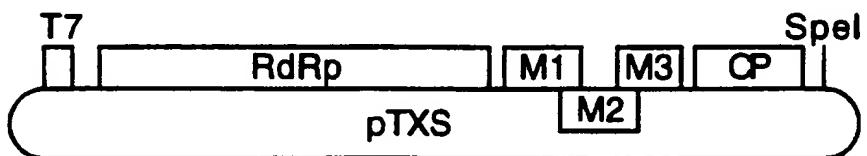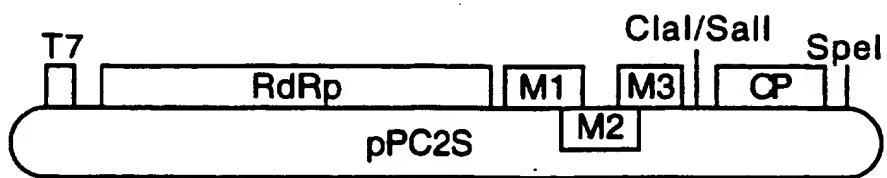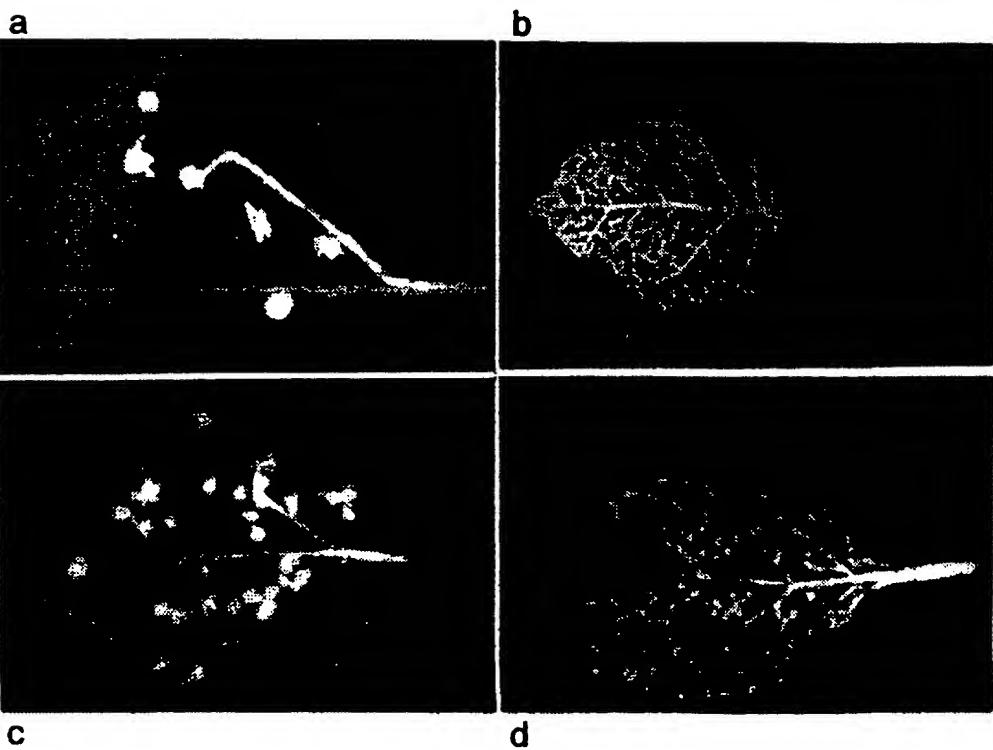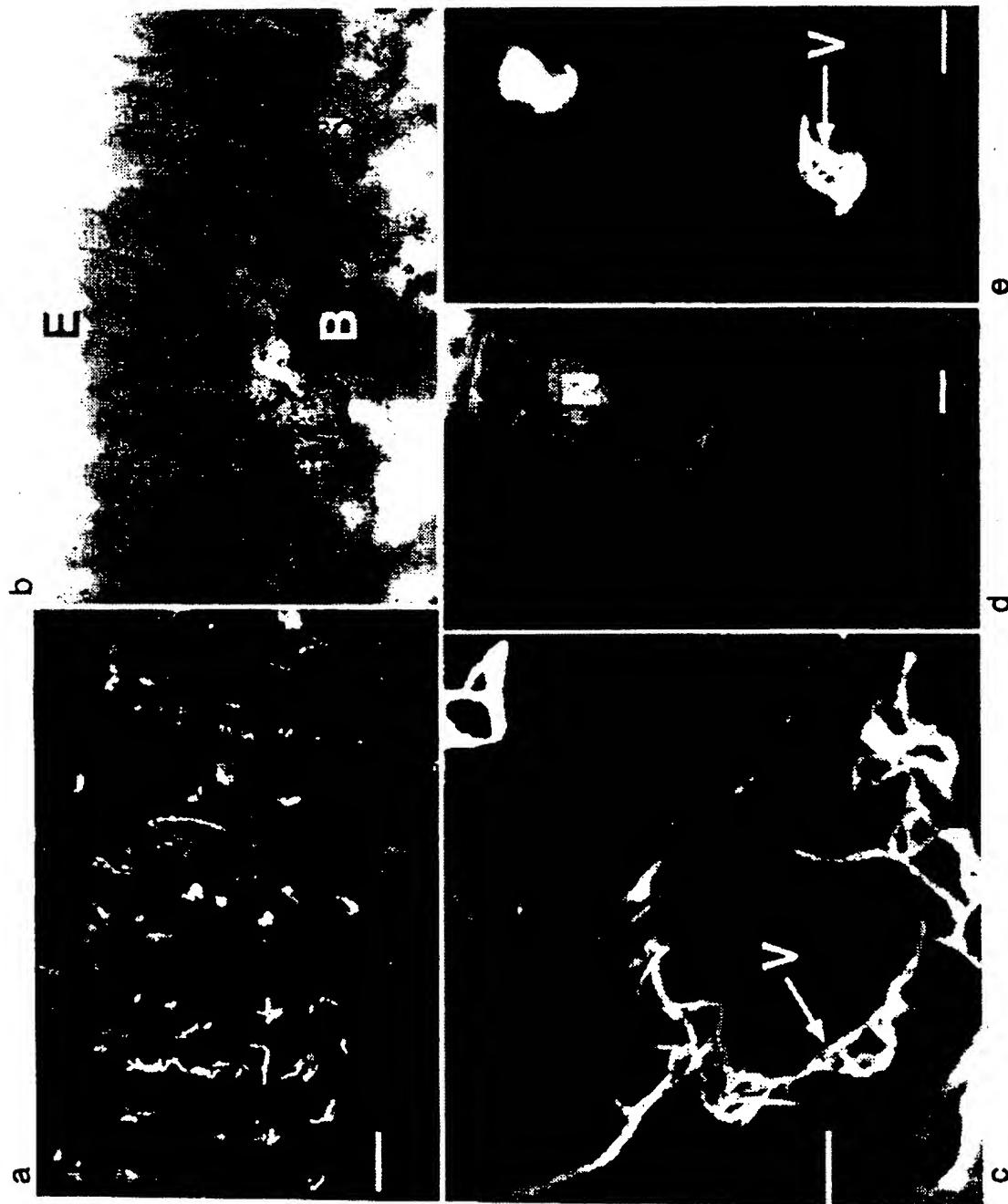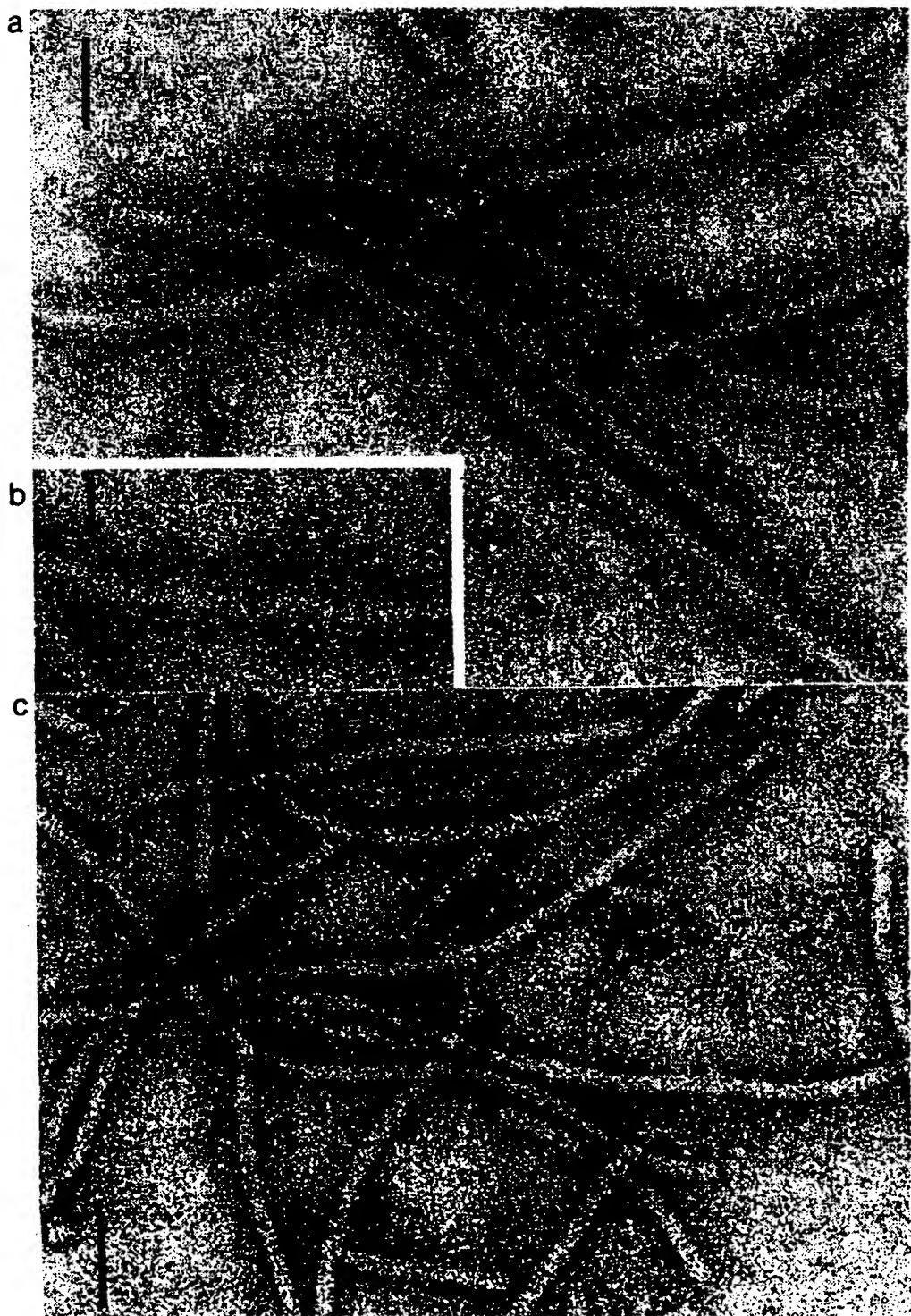
FIGURE 1b

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/62   C12N15/83   C07K14/08   C07K14/435   C12N7/01
         C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07K   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 15587 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 17 October 1991 see page 7, line 8 - line 22; example 4 see page 3, line 18 - page 4, line 27 --- -/--	1-3,8,9, 12,18, 20-23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
26 January 1996	15.03.1996

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Montero Lopez, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOTECHNOLOGY, v 1. 11, no. 10, October 1993 NEW YORK US, pages 1166-1170, MITTUR N. JAGADISH ET AL. 'High level production of hybrid Potyvirus-like particles carrying repetitive copies of foreign antigens in Escherichia coli' see abstract see page 1166, left column, paragraph 3 - page 1167, right column, paragraph 1 see page 1167, right column, paragraph 4 - page 1169, left column, paragraph 3 ---	1-3, 9-13, 20-23
X	WO,A,93 03161 (DONSON, JON ET AL.) 18 February 1993 see page 5, paragraph 2 see page 6, paragraph 3 - page 7, paragraph 3 see page 15, paragraph 2 - page 17, paragraph 2 see page 20, paragraph 3 - page 21, paragraph 1 see page 23, paragraph 2 - page 24, paragraph 1 see page 26, paragraph 5 - page 27, paragraph 3 see page 29, paragraph 2 - page 30, paragraph 1 see page 33, paragraph 2 - page 34, paragraph 1; examples 8,12 ---	1-24
X	DATABASE WPI Section Ch, Week 9013 Derwent Publications Ltd., London, GB; Class B04, AN 90-096520 'New plant virus RNA vector obtained by connecting foreign gene downstream of coat protein gene of tobamo virus RNA' & JP,A,02 049 591 (SUMITOMO CHEM IND KK) , 19 February 1990 see abstract ---	1,3,8,9, 11,20-23
X A	WO,A,92 18618 (AGRICULTURAL GENETICS COMPANY LIMITED) 29 October 1992 cited in the application see page 2, paragraph 2 - page 3, paragraph 1 see page 3, paragraph 3 - page 4, paragraph 1 see page 4, paragraph 3 - page 5, paragraph 3 see page 17, paragraph 2 - page 18, paragraph 5 ---	20-23 1-19,24

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Internation Application No
PCT/GB 95/02457

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VIROLOGY, vol. 197, no. 1, November 1993 ORLANDO US, pages 366-374, RAMAKRISHNAN USHA ET AL. 'Expression of an animal virus antigenic site on the surface of a plant virus particle'	20-23
A	 see abstract see page 366, left column, paragraph 1 - right column, paragraph 1 see page 368, left column, last paragraph - right column, paragraph 2 see page 370, right column, paragraph 1 - page 372, right column, paragraph 1 ---	1,2,9, 11-13,18
P,X	VACCINE, vol. 13, no. 12, August 1995 GUILDFORD GB, pages 1051-1057, JOHN FITCHEN ET AL. 'Plant virus expressing hybrid coat protein with added murine epitope elicits autoantibody response' see abstract see page 1051, right column, paragraph 2 - page 1052, left column, paragraph 1 see page 1053, right column, paragraph 1 - page 1054, right column, paragraph 2 see page 1055, left column, paragraph 1 - page 1056, right column, paragraph 1 -----	1-3,8,9, 11,12, 20-23

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

International Application No

PCT/GB 95/02457

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